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OYSTERS OF THE CONCH REPUBLIC (FLORIDA KEYS): A MOLECULAR PHYLOGENETIC STUDY OF *PARAHYOTISSA MCGINTYI*, *TESKEYOSTREA WEBERI* AND *OSTREOLA EQUESTRIS*

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ABSTRACT

We investigated the evolutionary relationships of three species of Florida Keys oysters, *Parahyotissa mcgintyi*, *Teskeyostrea weberi*, and *Ostreola equestris*, using nuclear and mitochondrial (mt) phylogenetic trees. Both 28S (nuclear) and 16S (mt) ribosomal gene trees consistently recovered a paraphyletic *Parahyotissa* in which *P. mcgintyi*, the type species, was robustly sister to a tip clade containing *P. numisma* and *Hyotissa hyotis*. This topology implies that there is no phylogenetic basis for *Parahyotissa* Harry, 1985, and we therefore recommend that all hyotissinid taxa be returned to the genus *Hyotissa* Stenzel, 1971. Phylogenetic placement of *T. weberi* within brooding oyster mt 16S gene trees conclusively demonstrated that it is a distinct ostreinid lineage, lacking any obvious candidate sister species, and falsified the hypothesis that it is a free-living ecomorph of the sponge commensal *Cryptostrea permollis*. Population-level mt COI sequence analysis of American *Ostreola equestris* and New Zealand *Ostrea aupouria* revealed that these two globally disjunct ostreinids, though remarkably close relatives, are reciprocally monophyletic sister taxa. Unlike a large fraction of the Floridian nearshore marine biota, *O. equestris* shows no evidence of a vicariant phylogenetic break distinguishing Gulf of Mexico and Atlantic populations. Our results imply that its present day Gulf/Atlantic distribution has been achieved by range extension from source Atlantic populations followed by a demographic growth pulse in the new Florida Keys/Gulf of Mexico habitats. *Ostreola equestris* individuals display an impressive range of shell morphs and coloration, some externally resembling *T. weberi*, and we present a plate of genotyped individuals that document this diversity.

Key words: Ostreidae, Gryphaeidae, systematics, biogeography, Florida, molecular phylogeny.

INTRODUCTION

The Florida Keys archipelago extends 362 km SW from the tip of peninsular Florida, separating Florida Bay from the Straits of Florida. This subtropical island chain represents the exposed surface layer of a much larger carbonate platform and has a rich bivalve fauna, estimated at approximately 325 species (Mikkelsen & Bieler, 2000). The strategic goal of the International Marine Bivalve Workshop, held at the Keys Marine Laboratory (Long Key) from 19–30 July 2002, was to expand our knowledge of targeted segments of this fauna. We elected to study the local oyster taxa, or at least that fraction accessible by wading, snorkeling and SCUBA diving during our limited sampling window.

Although oysters are among the most studied marine invertebrate taxa, their taxonomy and systematics is still fraught with uncertainty due to their xenomorphic post-larval growth patterns (Ranson, 1951; Quayle, 1988; Yamaguchi, 1994), relative dearth of tractable anatomical characters, and extensive anthropogenic global transfer (Dinamani, 1971; Edwards, 1976; Buroker et al., 1979; Chew, 1990; Carlton & Mann, 1996). Harry's (1985) ambitious taxonomic revision, based largely on morphology, represents the most recent comprehensive reclassification of living oysters. Subsequently, a number of de-

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tailed paleontological studies (Malchus, 1990; Malchus & Aberhan, 1998; Dhondt et al., 1999), together with a steady trickle of molecular phylogenetic analyses (Reeb & Avise, 1990; Littlewood 1994; Banks et al., 1993, 1994; Anderson & Adlard, 1994; Hare & Avise, 1998; Boudry et al., 1998; Ó Foighil et al., 1998; Jozefowicz & Ó Foighil, 1998; Ó Foighil & Taylor, 2000; Campbell, 2000; Steiner & Hammer, 2000; Lam & Morton, 2001; Giribet & Wheeler, 2002; Lapegue et al., 2002), have significantly refined our understanding of many aspects of ostreoidean evolution and systematics.

The ostreoidean fauna of the Florida Keys is atypical in that the ecologically dominant cupped oysters of the adjacent Caribbean and Atlantic seaboards are almost completely absent. Although isolated records occur in the Keys (Mikkelsen & Bieler, 2000), we did not encounter specimens of either the temperate *Crassostrea virginica* (Gmelin, 1791), or the tropical *C. rhizophorae* (Guilding, 1828) (Ostreidae, Crassostreinae). *Crassostrea virginica* populations are critically dependent on estuarine conditions, absent from the Keys, where salinity variation acts to reduce biotic competition and parasitism (Galtsoff, 1964; Ford & Tripp, 1996; Shumway, 1996).

Our sampling efforts yielded three distinct oyster groupings. By far the most common were small flat oysters (Ostreidae, Ostreinae), displaying an impressively diverse and overlapping range of shell morphology and coloration. Based on shell phenotype, many of these were readily identifiable as either *Ostreola equestris* (Say, 1834) or *Teskyostrea weberi* (Olsson, 1951); however, quite a few individuals were difficult to place with confidence. During dives, we encountered specimens of the gorgonian-associated *Dendostrea frons* (Linné, 1758) (Ostreidae, Lophinae) and the equally distinctive *Parahyotissa mcgintyi* Harry, 1985 (Gryphaeidae, Pycnodonteinae). We focused our efforts on the gryphaeid and flat oysters as they require the most systematic attention. In particular, we addressed the following four questions.

Systematic Placement of *Parahyotissa mcgintyi* Harry, 1985

Harry (1985) reorganized the gryphaeid (pycnodonteinid) tribe Hyotissini into the monotypic Indo-Pacific genus *Hyotissa* and a new genus *Parahyotissa* (containing three subgenera and four species) which includes the tropical Atlantic type species *P.* (*Parahyotissa*) *mcgintyi*, and the Indo-West-Pacific *P.* (*Numismoida*) *numisma* (Lamarck, 1819). He distinguished among the two hyotisssinid genera mainly by the relative degree of opening of the left promyal passage: open but reduced in *Hyotissa*, closed in *Parahyotissa*. We aimed to test the phylogenetic robustness of this generic reorganization by constructing nuclear and mitochondrial ribosomal gene trees incorporating these three taxa together with a neopycnodontinid gryphaeid, *Neopycnodonte cochlear* (Poli, 1795), that is sister to the Hyotissini (Ó Foighil & Taylor, 2000).

Phylogenetic Status of *Teskyostrea weberi*

Olsson (1951) considered *Ostrea weberi* to be the most distinctive regional species of oyster, and designated Key West as its type locatity. Harry (1985) supported its taxonomic distinctiveness, placing it in a monotypic new genus, *Teskeyostrea*. Alternatively, Abbott (1974) regarded *T. weberi* as a free-living ecophenotype, and junior synonym, of the sponge commensal *Cryptostrea permollis* (G. B. Sowerby II, 1871), and this taxonomic interpretation has been largely followed in the subsequent literature (Carriker & Gaffney, 1996). *Cryptostrea permollis* is recorded from the northeastern Gulf of Mexico and off North Carolina (Harry, 1985), and we did not encounter it in the Florida Keys. There are multiple records of *C. permollis* in the Florida Keys (Mikkelsen & Bieler, 2000); however, these refer to free-living, *T. weberi* (R. Bieler, pers. comm.). Jozefowicz & Ó Foighil (1998) incorporated, for comparative purposes, Keys specimens they identified as *T. weberi* in their molecular study of Southern Hemisphere flat oysters. However, they were unaware that the range of shell ecomorphs produced by another Keys ostreinid, *Ostreola equestris*, overlaps with that of *T. weberi*. Subsequent unpublished work by one of the authors (P. Baker) showed conclusively that the "*T. weberi*" specimens sequenced by Jozefowicz & Ó Foighil (1998) were actually *O. equestris*. The phylogenetic placement of *T. weberi* therefore still remains to be established. We revisited this issue by generating mitochondrial genotypes – large ribosomal subunit (16S) – from authentic *T. weberi* and incorporating them, together with *C. permollis* and *O. equestris* genotypes, into a phylogenetic analysis of brooding oysters.

Biogeographic Relationships of *Ostreola equestris* and *Ostrea aupouria* (Dinamani & Beu, 1981)

Jozefowicz & Ó Foighil (1998) uncovered a number of unexpectedly close phylogenetic relationships among geographically disjunct ostreinid taxa. Their Keys *Ostreola equestris* samples (misidentified as *Teskeyostrea weberi*, see above) differed from specimens of the New Zealand *O. aupouria* by as little as a single transversion in their mt 16S large subunit ribosomal gene fragments. We aimed to revisit this surprising biogeographic pairing by utilizing Cytochrome Oxidase I (COI), a faster-evolving mt gene fragment more useful in resolving oyster tip taxa (Ó Foighil et al., 1998), and by incorporating samples of *O. equestris* spanning the well-defined Gulf/Atlantic marine biogeographic break in southeastern Florida (Avise, 1992, 2000; Cunningham & Collins, 1994). In the absence of post-separation gene flow, the process of lineage sorting is expected to sequentially lead newly formed daughter populations from initial polyphyly, to paraphyly, and ultimately to reciprocal monophyly (Avise, 2000). We were interested in establishing whether these disjunct New Zealand/American populations were reciprocally monophyletic, or if one was a recent founder of the other. Another objective was to determine how the *aupouria/equestris* genetic disjunction scaled relative to the anticipated Gulf/Atlantic break in *O. equestris*. Two hypothetical topologies, each containing an *O. equestris* Gulf/Atlantic disjunction, are presented as exemplars in Figure 1. There are of course many other topological possibilities.

Shell Phenotype Variation in *Ostreola equestris*

Ostreola equestris is commonly known as the "crested" oyster and, as its informal name implies, it is described as having a shell with raised crenulated margins (Abbott, 1974). We encountered this morph in intertidal Keys habitat; however, subtidal individuals, genotyped in this study for mt markers, were usually cemented to the substratum along their entire left valves, yielding a very thin, contour-hugging, morph that exhibited a wide variety of coloration and sculptural texture, some of which closely approximated the *Teskeyostrea weberi* phenotype (Olsson, 1951; Harry, 1985). Employing genotyped individuals only, we aimed to give a photographic summary of the impressive range of shell phenotypes displayed by our samples of this species.

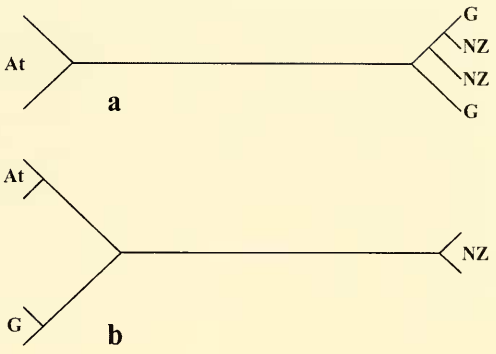

FIG. 1. Two exemplary unrooted mitochondrial tree topologies predicted by distinct hypotheses of historical relationships among geographically disjunct sister populations of New Zealand (*Ostrea aupouria*) and American (*Ostreola equestris*) ostreinids. Both hypotheses assume *a priori* that *O. equestris* has undergone cladogenesis into distinct Atlantic (At) and Gulf (G) lineages, a well-documented pattern among coastal Floridian marine taxa (Avise, 1992, 2000; Cunningham & Collins, 1994). There are of course many other hypothetical topologies that could be entertained. a, *O. aupouria* (NZ) represents a recent founder population of Gulf *O. equestris* (G) and genotypes of the former are predicted to nest within a Gulf tip clade; b, *O. aupouria* (NZ) has experienced a distinct evolutionary history that predates the origin of the Gulf/Atlantic disjunction in *O. equestris* and all three groupings are predicted to be reciprocally monophyletic with the stem branch leading to *O. aupouria* (NZ) being the most pronounced.

MATERIALS AND METHODS

A summary of sampling locations and of voucher specimen information is outlined in Table 1, and specific sampling details for Floridian taxa are given in the following paragraphs. For specimens collected in the Florida Keys, all collections were made via snorkeling in depths from 1–5 m, except collections from IMBW-FK-650 where SCUBA was used to sample specimens from roughly 30 m. These specimens were preserved in 95% denatured alcohol and then transferred to 95% non-denatured alcohol upon return to the Department of Malacology at the Florida Museum of Natural History. Specimens collected elsewhere were sampled from shore and preserved in ≥ 70% ethanol.

TABLE 1. Species identification and sampling locality data, together with voucher specimen information. UMMZ and FLMNH numbers respectively refer to the voucher specimen catalog numbers of the Mollusk Division, University of Michigan Museum of Zoology, and the Department of Malacology, Florida Museum of Natural History. See Mikkelsen & Bieler (2004) for specific details concerning the International Marine Bivalve Workshop (IMBW-FL) sampling stations.

Taxa	Location	# of individuals sequenced	Catalog #
Family Gryphaeidae			
Subfamily Pycnodonteinae			
Parahyotissa mcgintyi	IMBW-FK-650	1	UMMZ 300092
Parahyotissa numisma	Guam	1	UMMZ 265996
Hyotissa hyotis	Guam	1	UMMZ 265995
Neopycnodonte cochlear	Maui, Hawaii	1	UMMZ 265997
Family Ostreidae			
Subfamily Ostreinae			
Teskeyostrea weberi	IMBW-FK-645	4	FLMNH 298644
Ostreola equestris	IMBW-FK-629	9	FLMNH 298643
Ostreola equestris	IMBW-FK-644	1	FLMNH 298645
Ostreola equestris	IMBW-FK-649	1	FLMNH 298640
Ostreola equestris	Skidaway River, Georgia	11	UMMZ 300093
Ostreola equestris	Cedar Key, Florida	10	UMMZ 300094
Ostrea aupouria	Hauraki Gulf, New Zealand	12	UMMZ 255404
Cryptostrea permollis	Panacea, Florida	2	UMMZ 255410
Subfamily Crassostreinae			
Crassostrea virginica	Skidaway River, Georgia	3	UMMZ 300095
Crassostrea virginica	Panacea, Florida	2	UMMZ 300096

Parahyotissa mcgintyi

Numerous specimens of the gryphaeid *Parahyotissa mcgintyi* were sampled (by L. Kirkendale and G. Steiner) from the superstructure epibenthos of the sunken vessel *Thunderbolt* (IMBW-FK-650; Table 1) – apparently this species' first record from the Florida Keys (Mikkelsen & Bieler, 2000). *Parahyotissa mcgintyi* is easily distinguished from other regional oysters by its frequently plicated shell margins, absence of clasper spines, typically pycnodonteinid vesicular shell structure (Fig. 2), and presence (in live adult specimens) of a bright orange pigment in ovarian tissue (Harry, 1985). In order to test Harry's (1985) taxonomic rearrangement of the Hyotissini, we sequenced a 941nt (post-alignment length) fragment of nuclear 28S rDNA, added it to Ó Foighil & Taylor's (2000) homologous 28S ostreoidean matrix, and analyzed the resulting dataset utilizing pterioid outgroups (Giribet & Distel, 2003). A complementary gryphaeid mt 16S rDNA data set was constructed and then phylogenetically analyzed using *Neopycnodonte cochlear*, a sister taxon to the Hyotissini (Ó Foighil & Taylor, 2000), as an outgroup.

Teskeyostrea weberi

Specimens of *Teskeyostrea weberi* were recovered (by L. Kirkendale) from one of our sampling sites: the ocean-side shore of Grassy Key (IMBW-FK-645; Table 1), where it was locally abundant attached to the underside of large boulders at depths of 1–3 m. Positive identification of this species was made not only on the basis of its shell characters – flat, thin apricot-colored shell ornamented with fine radial ribbing and thin lamellose extensions (Olsson, 1951; Harry, 1985) – but also on its lack of an anal appendage, a prominent anatomical feature of *Ostreola equestris* (Harry, 1985). To place *Teskeyostrea weberi* phylogenetically, we generated mt 16S sequences for four individuals, yielding two haplotypes, which were incorporated into Jozefowicz & Ó Foighil's (1998) brooding oyster 16S matrix. This matrix was further supplemented by 16S sequences (two haplotypes) generated from 11 Florida Keys *Ostreola equestris* specimens sampled from three locations in the Florida Keys (Table 1). These latter specimens collectively displayed a wide variety of shell morphs, including *T. weberi* look-alikes, but exhibited

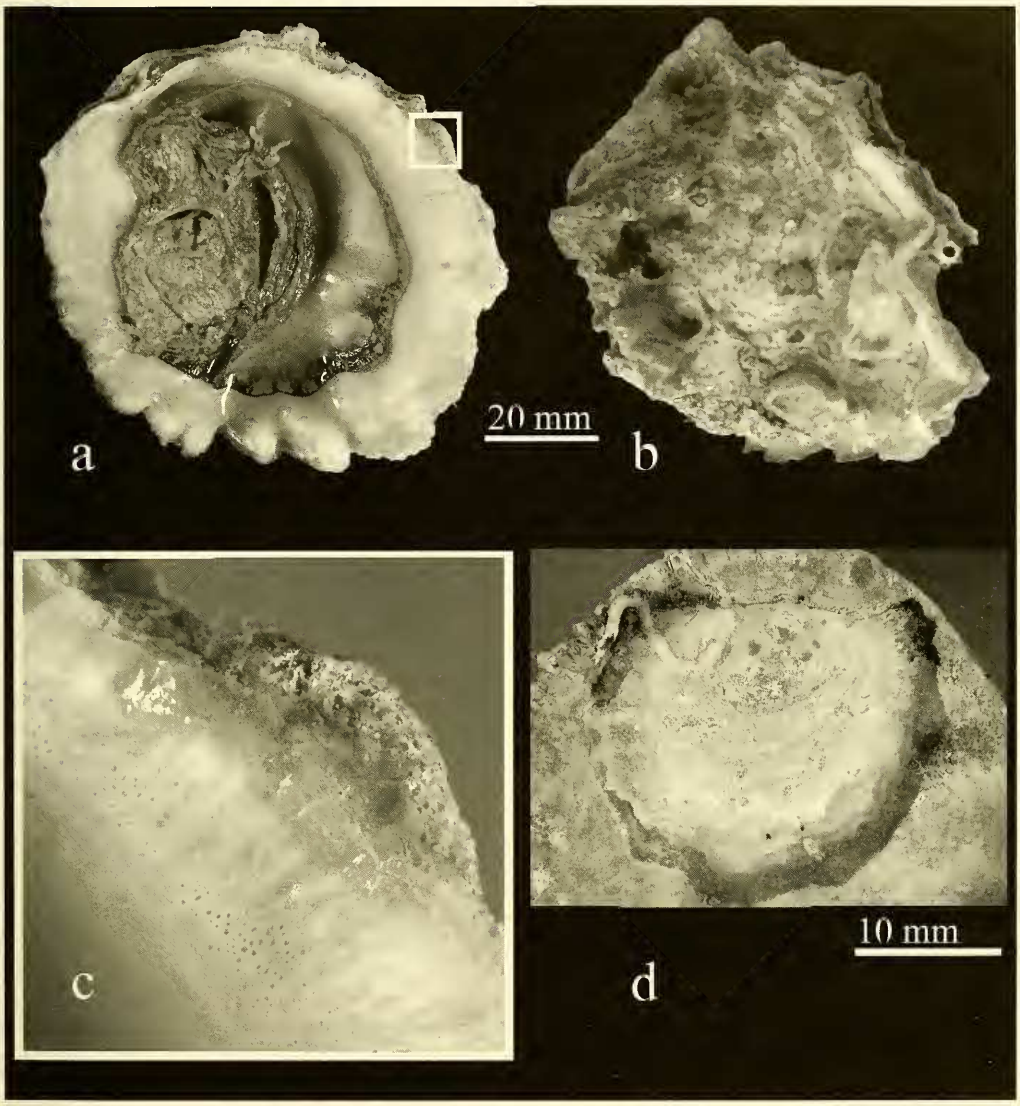

FIG. 2. Views of gross shell morphologies of adult and juvenile *Parahyotissa mcgintyi* specimens sampled from IMBW-FK-650. a, internal view of the left valve of an adult preserved in 95% ethanol after shucking (Note the prominent plication of the ventral valve margin); b, external view of the right valve of specimen depicted in 2a (Note the heavy fouling which obscures the valve outline); c, detail of anterio-dorsal inner edge of left valve of adult (see boxed area in 2a) showing the distinctive vesicular substructure characteristic of pycnodonteinid gryphaeids (Harry, 1985); d, external view of intact juvenile (note straight hinge line, flattened D-shaped profile and the vesicular substructure evident in abraded surface areas).

distinct anal appendages (mainly digitform, some more cardiform in outline). Finally, we added to the single available 16S haplotype of the sponge commensal *Cryptostrea permollis* by sequencing two additional specimens (Table 1).

Ostreola equestris

In order to more fully resolve the phylogenetic relationships of these geographically disjunct, polytomous (at least for 16S, Fig. 4), New Zealand/American tip taxa, a mt COI

gene fragment (626 nt) data set was generated for a total of 44 individual oysters. Twelve New Zealand *Ostrea aupouria* – reliably distinguished by their possession of an anal appendage (Dinamani & Beu, 1981) from the co-occurring *Ostrea chilensis* (Philippi, 1844) – were sequenced, yielding 6 haplotypes, as were 32 *Ostreola equestris* specimens which collectively contained 15 haplotypes.

We were interested in establishing if *Ostreola equestris* exhibits a regional Gulf/Atlantic genetic break in southeastern Florida in common with many other co-occurring nearshore marine taxa (Avise, 1992, 2000; Cunningham & Collins, 1994) and, if so, how it might scale relative to the *equestris*/*aupouria* disjunction. In addition to Florida Keys specimens (N = 11, six haplotypes), our 32 *O. equestris* individuals sequenced for COI also included specimens from the northeastern Gulf of Mexico (Cedar Key, N = 11, seven haplotypes) and from the Atlantic coast of Georgia (Skidaway River estuary, N = 10, six haplotypes). To provide a phylogeographic yardstick, we also generated homologous COI sequences (598 nt) for a token number of replicate Gulf (Panacea, Florida Panhandle, N = 2, one haplotype) and Atlantic (Skidaway River, N = 3, 2 haplotypes) specimens of the cupped oyster *Crassostrea virginica*. This ecologically dominant regional oyster species displays a well-characterized Gulf/Atlantic mt disjunction centered on southeastern Florida (Reeb & Avise, 1990).

Molecular Methods

Specimens utilized in this study were processed for molecular characterization either at the University of Florida (by L. Kirkendale) or the University of Michigan (by T. Lee). As a result, there were some minor methodological distinctions associated with DNA template preparation and PCR amplification as referred to below. All novel DNA sequences were generated at the University of Michigan's DNA Sequencing Core and have been deposited in GenBank (Accession #s AY376596–AY376635).

Genomic extractions and amplifications of flat oyster samples collected during the Florida Keys Bivalve Workshop were conducted by L. Kirkendale at the Florida Museum of Natural History Molecular Phylogenetics Lab at the University of Florida (UF). Total genomic DNA was obtained from ethanol-preserved mantle tissue using modifications of standard protocols. Roughly 20–30 mg of tissue was finely cut, ground with a mortar and pestle and placed in 750 µL of DNAzol with 5–20 µL of 5–20 mg/ml proteinase K (Molecular Research Center, Inc.). Tissue was gently shaken overnight on an orbital shaker and following three rounds of ethanol extraction and centrifugation, the pellet was eluted in 100 mL ddH20 (for further details of DNAzol extraction procedure, refer to Chomczynski et al. 1997). Universal primers were used to amplify 16S and COI gene regions sequenced from the above-mentioned samples and were as follows: 16Sar 5'-CGCCTGTTTATCAAAAACAT-3' and 16Sbr 5'-GCCGGTCTGAACTCAGATCACGT-3' (Kessing et al. 1989) and LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et al., 1994). Reactions included 1µL of genomic DNA template and 31.8 µL ddH2O, 5 µL of 10X TAQ PCR buffer (Perkin Elmer), 5 µL of dNTPS (10 mM stock), 2 µL of each primer (10 µM stock), 3 µL of MgCl2 solution (25 mM stock, Perkin Elmer) and 0.2 µL TAQ enzyme (Perkin Elmer). Reactions for 16S were initially denatured at 96°C for 150 sec, followed by 37 cycles of 94°C for 40 sec, 52°C for 35 sec, and 72°C for 60 sec. Reactions for COI were handled similarly except that the initial denaturation step was at 95°C for 120 sec and that 40 cycles of amplification were employed with a 40°C annealing temperature. All amplifications were run with positive and negative (no template) controls. PCR products were visualized by electrophoresis on 1% TBE agarose gels, stained with ethidium bromide solution and photodocumented. Successful PCR products were cleaned for cycle sequencing using Wizard PCR Preps (Promega), following described protocols. Verification of the cleaned PCR product occurred in the same manner as for initial PCR products.

Ostreola equestris samples from Cedar Key were extracted at UF, as above, but amplified at the Museum of Zoology, University of Michigan (UMMZ), by T. Lee, along with Skidaway River *O. equestris* samples, using specifically designed COI primers: 5'-GATATTGGACGGTTTTATAT-3' and 5'-CCAAAAATCAAAACAATGCT-3' (Lee, unpublished). DNA template preparation methods utilized at the UMMZ are detailed in Lee & Ó Foighil (2003). Other target gene fragments amplified at the UMMZ were mt 16S from *Cryptostrea permollis* and from the four

gryphaeid study species (Table 1) using Kessing et al. (1989) primers, 28S nuclear ribosomal domains 1–3 from *Parahyotissa mcgintyi* using Ó Foighil & Taylor's (2000) primer set, and mt COI from *Ostrea aupouria*, and *Crassostrea virginica* Gulf (Panacea) and Atlantic (Skidaway River) samples using Folmer et al. (1994) primers. A touchdown (Palumbi, 1996) protocol was used for all UMMZ PCR reactions [after 4 min denaturation at 94°C, the initial annealing temperature of 65°C was decreased by 2°C/cycle (40 sec denaturing at 94°C, 40 sec annealing and 1.5 min extension at 72°C) until the final annealing temperature (45°C for COI, 50°C for 16S and 52°C for 28S) was reached and subsequently maintained for an additional 30 cycles].

Phylogenetic Methods

Initial alignments were constructed using Clustal X (Thompson et al., 1997) using default parameters and then adjusted by eye to minimize mismatches in the ribosomal gene datasets. Phylogenetic analyses were conducted on each of six molecular datasets – (1) gryphaeid 28S, (2) gryphaeid 16S, (3) Ostreid/Lophinid 16S, (4) *Ostrea aupouria/ Ostreola equestris* COI, (5) *O. equestris* COI, and (6) *Crassostrea virginica* COI – under the maximum parsimony (MP) optimality criterion using PAUP*4.0b10 (Swofford 2002). While unrooted anayses were performed on COI datasets, the pterioid taxa, *Neopycnodonte cochlear*, and lophinid taxa were designated as outgroup for gryphaeid 28S, gryphaeid16S and ostreid 16S datasets respectively. MP analyses were performed using heuristic search option with 100 random stepwise additions and tree bisection-reconnection (TBR) branch-swapping. Gaps were treated as a missing state, character states were treated as unordered and equal weights were assumed. Branch support was estimated by bootstrapping (Felsenstein, 1985) (500 replicates, heuristic searches, 10 random additions each) and decay indices (Bremer, 1994), generated in TreeRot (Sorenson, 1996).

We wished to construct unrooted gene networks for three COI datasets (*Ostreola equestris* and *O. aupouria*; *O. equestris* alone, *Crassostrea virginica* alone) and took a Maximum likelihood (ML) approach because two of the three (*O. equestris* and *O. aupouria*; *O. equestris* alone) produced multiple equally most parsimonious trees. A MP tree was first used to estimate the log-likelihood scores using PAUP*. The best-fit ML model for each partition was then determined by hierarchical likelihood ratio tests (hLRTs) using Modeltest 3.06 (Posada & Crandall, 1998). ML analyses were conducted using a heuristic search option in which the parameter values under the best-fit model were fixed and a MP tree was used as a starting point for TBR branch swapping. The K81uf model [K81 model (Kimura, 1981) with unequal base frequencies] + Γ [gamma-distributed heterogeneity of the substitution rate across sites (Yang, 1994)] was chosen as the best-fit model for the combined *Ostreola equestris* and *O. aupouria* dataset. For the *O. equestris* and *C. virginica* COI datasets, the respective best-fit models chosen were K81uf and HKY (Hasegawa et al., 1985).

RESULTS

Systematic Placement of *Parahyotissa mcgintyi*

Figure 3 shows the most parsimonious gene tree obtained when a *P. mcgintyi* 28S genotype was added to, and analyzed with, Ó Foighil & Taylor's (2000) ostreoidean 28S dataset. We obtained a paraphyletic *Parahyotissa* and a robust terminal sister relationship for the two Pacific Hyotissini: *P. numisma* and *Hyotissa hyotis*. A congruent topology was recovered when the 16S sequences for the four gryphaeid taxa at our disposal (Table 1) were subjected to a maximum parsimony analysis (Fig. 3). The earlier study (Ó Foighil & Taylor, 2000) should be consulted for a detailed discussion of the ostreid clade topology.

Phylogenetic Status of *Teskyostrea weberi*

Figure 4 shows the strict consensus topology of the 54 most parsimonious trees obtained when the brooding oyster 16S matrix was analyzed using the lophine taxa as outgroups. Major elements of the topology are congruent with that obtained, and discussed at length, in an earlier study (Jozefowicz & Ó Foighil, 1998) and will not be reiterated here. The salient features of the topology concern the relative placement of the three Floridian flat oyster taxa (labeled in bold text). All three occur in distinct, well-supported terminal clades: *Teskeyostrea weberi* on its own, *Ostreola equestris* in a terminal polytomy with the New Zealand *Ostrea*

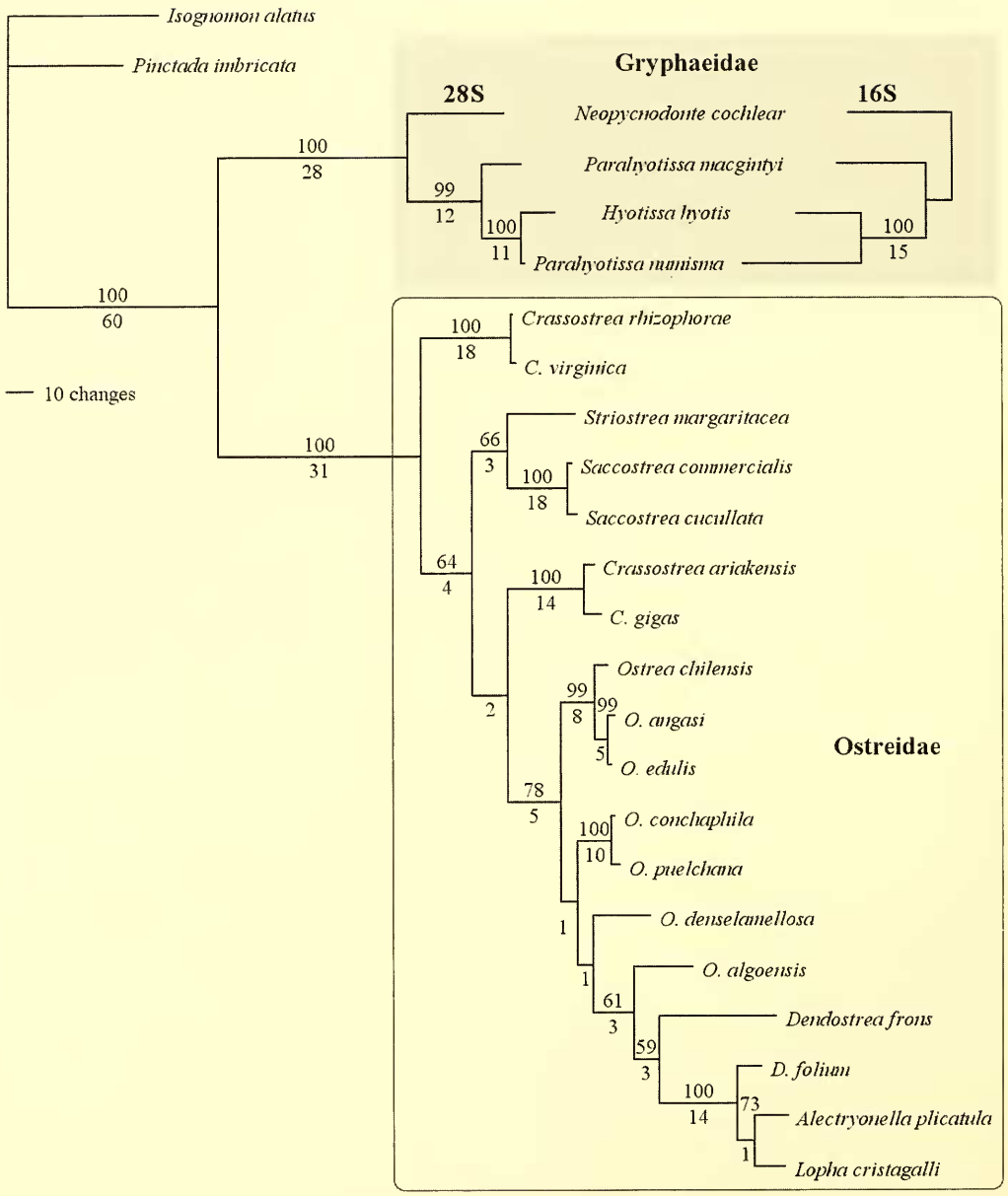

FIG. 3. The single most parsimonious tree (809 steps, CI = 0.668, RI = 0.779) obtained by heuristic unweighted searches of 28S genotypes for 22 oyster taxa, including 4 gryphaeid species, with the two pterioids, *Pinctada* and *Isognomon*, designated as outgroups. See also the juxtaposed single most parsimonious tree (173 steps, CI = 0.948, RI = 0.710) obtained by heuristic unweighted searches of gryphaeid mt 16S genotypes, in which *Neopycnodonte cochlear* was the designated outgroup. Numbers above the branches represent bootstrap values (> 50) and numbers below indicate decay index values.

aupouria, *Cryptostrea permollis* in a terminal polytomy with the Argentine *Ostrea puelchana*. A prominent basal ostreinid (+ *Dendostrea frons*) polytomy captures the branch supporting the *T. weberi* tip clade (Fig. 4), thereby obscuring its sister relationships.

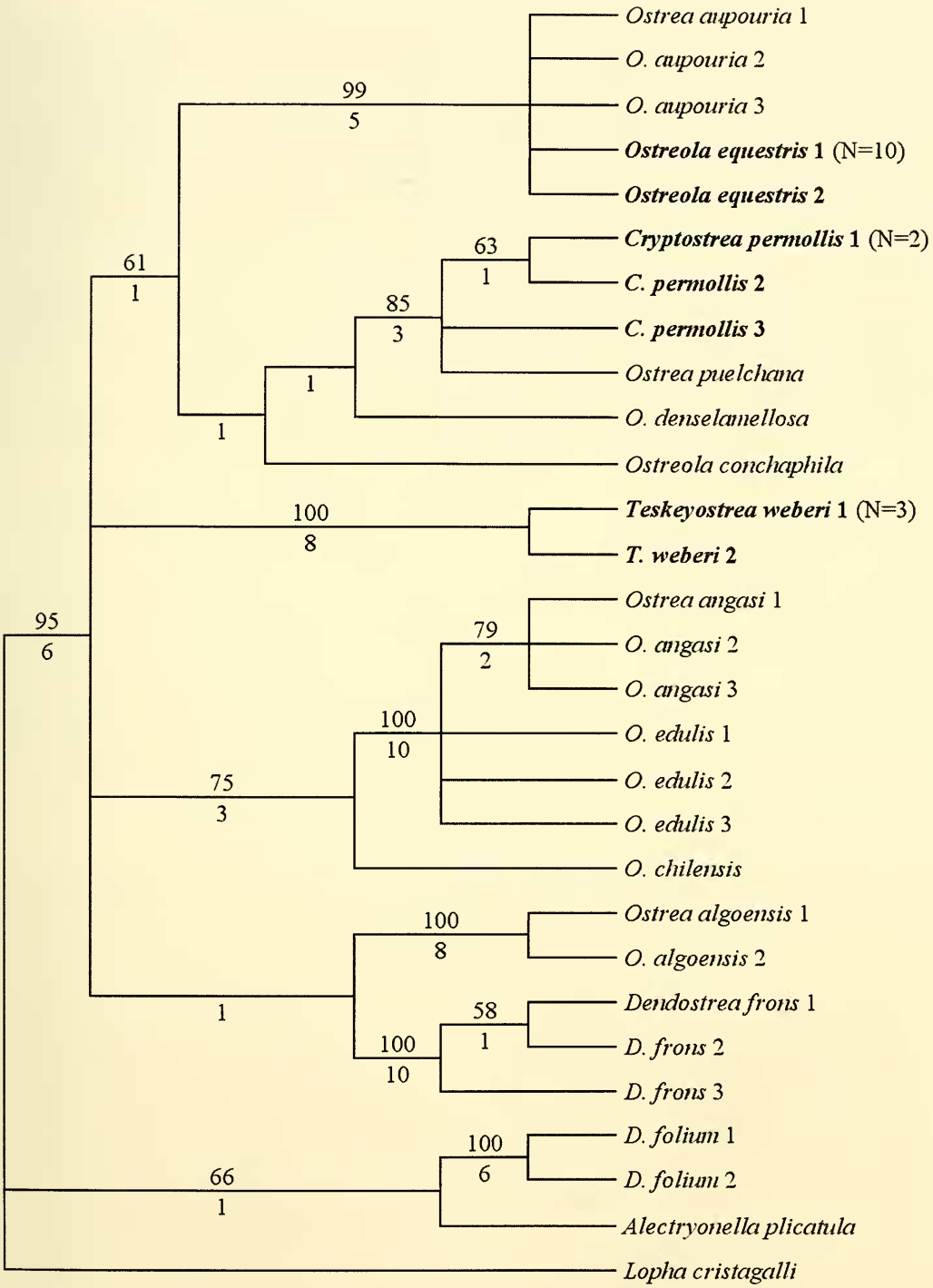

FIG. 4. Strict consensus of 54 equally most parsimonious trees (174 steps, CI = 0.6379, RI = 0.8437) resulting from heuristic unweighted searches of 29 brooding oyster 16S genotypes. The lophine taxa *D. folium*, *D. frons*, *A. plicatula* and *L. cristagalli* were designated as outgroups. Florida Keys ostreinid taxa are in boldface. Bootstrap values (> 50) and decay indices are shown above and below the branches, respectively.

Biogeographic Relationships of *Ostreola equestris* and *Ostrea aupouria*

A maximum-likelihood analysis of the combined American *Ostreola equestris* and New Zealand *Ostrea aupouria* COI dataset is shown as an unrooted network in Figure 5. New Zealand and American samples were reciprocally, and robustly, monophyletic. Note however, that the minimum cumulative branch lengths separating members of the two clades was less than that of the maximum branch lengths separating within-clade *O. equestris* haplotypes.

Figure 6 concerns only American taxa and shows the unrooted maximum-likelihood Gulf/Atlantic COI networks for both *Ostreola equestris* and *Crassostrea virginica*. The *Crassostrea virginica* Gulf/Atlantic phylogenetic split, estimated by Reeb & Avise (1990) from whole mt genome RFLP assays at approximately 2.5% divergence, was also recovered from our token sample of Gulf/Atlantic CO I gene fragment sequences (1.8%; 11 substitutions over 598 nt). In sharp contrast, no such disjunction was evident in *Ostreola equestris*. Two haplotypes were found in all three regional populations (Table 2, Fig. 6), including by far the most common mt COI genotype (AFG1; N = 13). This latter mt genotype was numerically predominant in both Gulf (Cedar Key, 6/11) and Florida Keys (5/11) samples of *Ostreola equestris*, but not among our Atlantic (Skidaway River sample; 2/10) specimens. If we consider the former two samples in isolation, the numerically predominant haplotype was centrally placed and connected to all but one (F4) of the

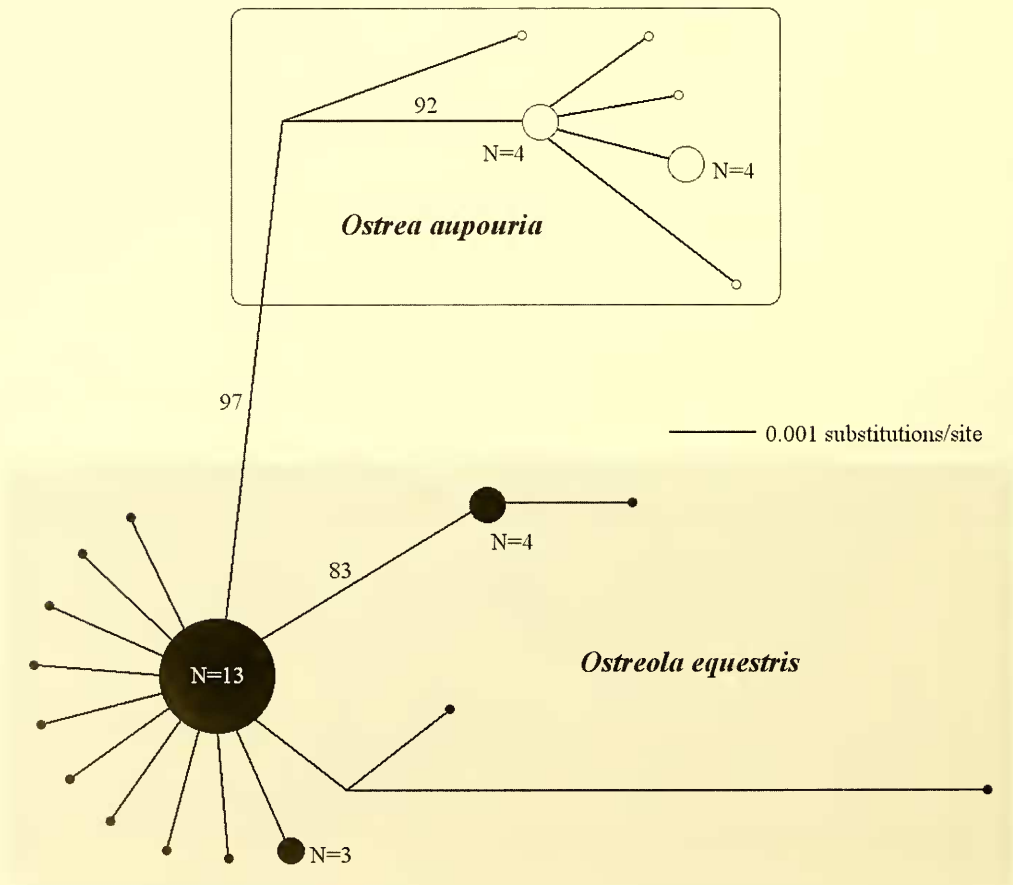

FIG. 5. Maximum likelihood network (-ln = 1073.4044) of *Ostrea aupouria* (New Zealand) and *Ostreola equestris* (American) COI haplotypes. Numbers on the branches are MP bootstrap values.

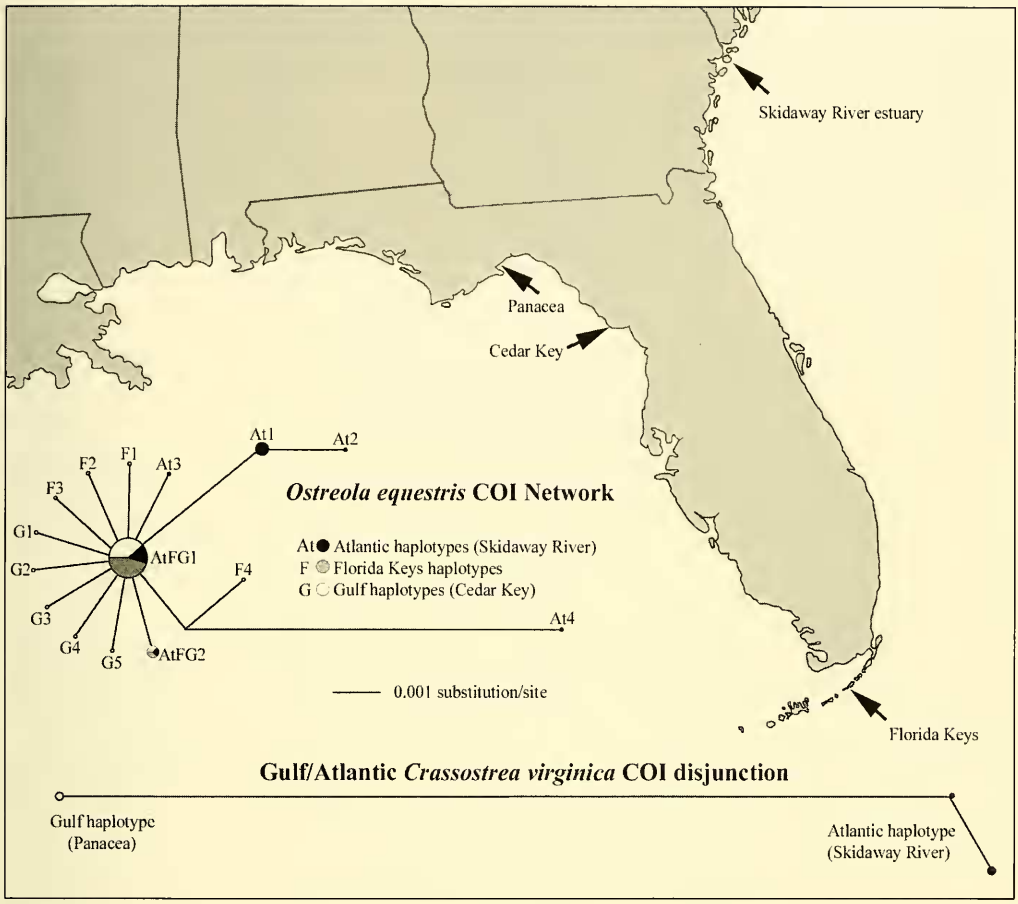

FIG. 6. Regional map showing our collection sites for Gulf/Atlantic *Ostreola equestris* and *Crassostrea virginica* samples and also the superimposed maximum likelihood networks of the resulting *O. equestris* (-ln = 985.5091) and *C. virginica* (-ln = 878.0842) COI haplotypes.

TABLE 2. Relative distribution of the 16 COI genotypes recovered from the three regional Gulf/Atlantic *Ostreola equestris* sampling locations. The prefixes At, F, G and AtFG, respectively indicate haplotypes found solely in the Atlantic (Skidaway River) site, solely in the Florida Keys sites, solely in the Gulf (Cedar Key) site, and finally, those recovered from all three sites. See Figure 6 for map showing sampling site locations and the inferred topological relationships among the COI haplotypes.

	AtFG1	AtFG2	At1	At2	At3	At4	F1	F2	F3	F4	G1	G2	G3	G4	G5	G6
Skidaway River	2	1	4	1	1	1	-	-	-	-	-	-	-	-	-	-
Florida Keys	6	1	-	-	-	-	1	1	1	1	-	-	-	-	-	-
Cedar Key	5	1	-	-	-	-	-	-	-	-	1	1	1	1	1	1

other 10 COI genotypes recovered from the Gulf (Cedar Key) and Florida Keys populations by single substitutions (Fig. 6). Our Atlantic (Skidaway River) sample exhibited a different topological pattern characterized by a relatively extensive network in which the constituent haplotypes showed more pronounced collective phylogenetic definition (Fig. 6).

Shell Phenotype Variation in *Ostreola equestris*

An impressive diversity of *O. equestris* shell phenotypes was recovered from the Florida Keys, and indeed also from single sampling sites, such as the Summerland Key Horseshoe. Intertidal Horseshoe specimens exhibited a shell morphology that is typically associated with this species: gray oval shells with raised crenulated margins (Abbott, 1974). Figure 7a shows a cluster of specimens showing this morphology, sampled in this particular case from the Skidaway River study population. Subtidal Florida Keys specimens were generally flatter in appearance, in some cases markedly so, and frequently incorporated a diversity of pigmentation colors and patterns, some of which are presented in Figure 7 (b–f). Exemplars spanning the range of *O. equestris* shell phenotypes found in the Horseshoe site, and other locations in the Keys, were genotyped using mt (16S and COI) markers and no evidence for genetic differentiation was evident among them. A minority of *O. equestris* individuals displayed shell phenotypes that resembled *Teskeyostrea weberi* in external appearance: very thin shells with golden brown pigmentation sculptured with fine radial ribbing and lamellose extensions (Fig. 7).

DISCUSSION

Systematic Placement of *Parahyotissa mcgintyi*

Our nuclear and mt ribosomal gene trees consistently recovered a paraphyletic *Parahyotissa* in which *P. mcgintyi*, the type species, was robustly sister to a tip clade containing *P. numisma* and *Hyotissa hyotis*. This topology implies that the character state used by Harry (1985) to distinguish *Parahyotissa* (closed left promyal passage) is plesiomorphic in extant Hyotissini, rather than a synapomorphy diagnosing a *Parahyotissa* clade, and that the condition in the monotypic genus *Hyotissa* (open but reduced left promyal passage) is autapomorphic. Based on available information, there seems to be no phylogenetic basis for Harry's *Parahyotissa*. Future research incorporating *P.* (*Parahyotissa*) *imbricata* (Lamarck, 1819) and *P.* (*Pliohyotissa*) *quercinus* (G. B. Sowerby II, 1871), may uncover more than one natural (i.e., monophyletic) group within the Hyotissini that can be defined by morphological synapomorphies and warrant generic status. Until then, we recommend that all hyotissinid taxa be returned to the genus *Hyotissa* Stenzel, 1971.

Phylogenetic Status of *Teskeyostrea weberi*

Our 16S strict consensus tree topology (Fig. 3) conclusively demonstrates that this species is not a free-living ecomorph of the sponge commensal *Cryptostrea permollis*, as thought by Abbott (1974), but is instead a distinct ostreinid lineage lacking (at present) any obvious candidate sister species. Olsson (1951) had proposed the eastern Pacific "*Ostrea iridescens*", synonymized with *Striostrea prismatica* (Gray, 1825) by Harry (1985), as a putative sister species to *T. weberi*, based on the similarity of the former's juvenile shell phenotype to that of the adult *T. weberi*. However, *S. prismatica*'s taxonomic placement in the cupped oyster subfamily Crassostreinae (Harry, 1985), which is supported by preliminary molecular data (Lee & Ó Foighil, unpublished), rules this out. A more comprehensive sampling of brooding oyster global diversity, including data from genes other than 16S, is required to better resolve *T. weberi*'s phylogenetic position within the Ostreinae/Lophinae.

Although *Teskyostrea weberi* and *Ostreola equestris* represent very distinct lineages (Fig. 3), they co-occur in the Florida Keys, and a fraction of latter species resemble *T. weberi* in their external appearance (Fig. 7). Fortunately, these *O. equestris weberi*-lookalikes can be distinguished upon dissection by their distinct anal appendage (Harry, 1985), and their relatively larger adductor muscle. Based on our preliminary observations, there may also be ecological and larval settlement differences among these two ostreinid taxa in the Florida Keys. All of the *T. weberi* specimens we encountered were attached to the underside of rocks (Harry, 1985: fig. 25) in an oceanside location, whereas *O. equestris* were commonly sampled from the exposed hard surfaces in bayside locations.

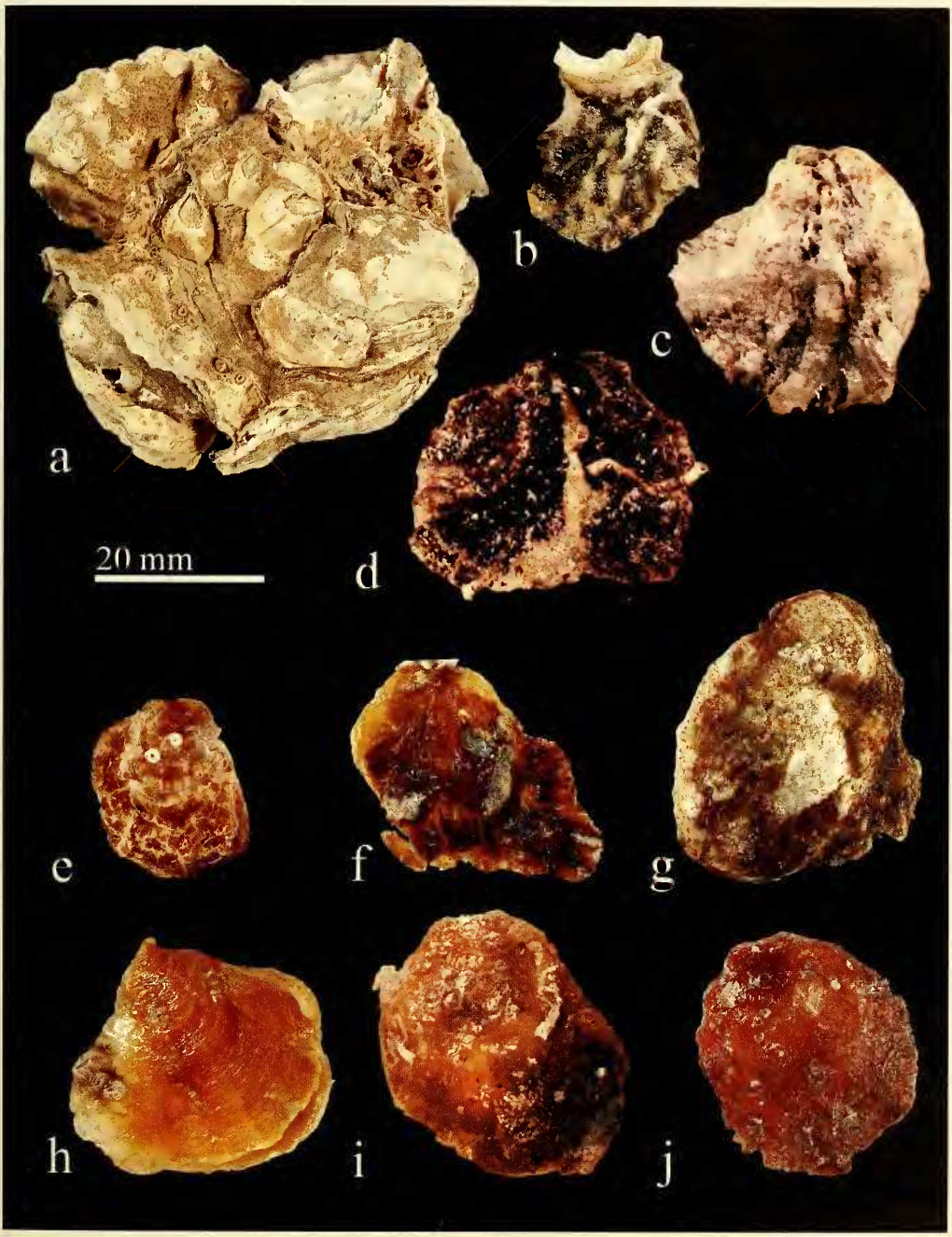

FIG. 7. Shell phenotypes. a–f, displayed by genotyped *Ostreola equestris* sampled from the Skidaway River, Georgia (a, cluster of individuals), and from 2 sites in the Florida Keys (b–e, IMBW-FK-629 from rock surfaces and f, IMBW-FK-649 epifaunal on *Pinna*); g, a specimen of *Ostrea aupouria*, New Zealand sister species of *Ostreola equestris* (UMMZ 255404); h, a specimen of the sponge commensal *Cryptostrea permollis* from Panacea, Florida Gulf Coast (UMMZ 255410); i and j, individuals of *Teskeyostrea weberi* sampled from IMBW-FK-645.

Biogeographic Relationships of *Ostreola equestris* and *Ostrea aupouria*

The COI gene tree topology (Fig. 5) demonstrates that our respective study populations of New Zealand *Ostrea aupouria* and Gulf/Atlantic *Ostreola equestris* are reciprocally monophyletic. This result is sufficient, at least for now, for retention of their respective specific status. Coan et al. (2000) rejected the separation of *Ostreola* from *Ostrea* based on morphological characters and the phylogenetic validity of Harry's (1985) *Ostreola* is questionable given that two of his three constituent species (*O. equestris* and *O. conchaphila*) are not sister taxa in our gene trees (Fig. 3). However, a definitive generic designation for *equestris* and *aupouria* requires data from the Mediterranean/African-Atlantic type species *Ostreola stentina* (Payraudeau, 1826).

Two lines of evidence indicate that the *Ostreola equestris*/*O. aupouria* disjunction results from evolutionarily recent dispersal rather than ancient vicariance. Maximum within-population COI genetic divergence for the Skidaway River sample exceeds the minimum New Zealand/American divergences obtained (Fig. 5). This result implies that the age of the *O. equestris*/*O. aupouria* disjunction may be less than the haplotypic lineage sorting time window for the Atlantic population of the *O. equestris*. Although we do not have a fossil-calibrated lineage-specific clock for any oyster, the well-studied Gulf/Atlantic *Crassostrea virginica* divergence has been dated, using "conventional calibrations" to approximately 1.2 myr (Reeb & Avise, 1990). Parsimony analysis of our token samples of Gulf/Atlantic *C. virginica* COI sequences found that they differed by 11 steps (1.83% of the 598 nt fragment). The minimum number of substitutions separating the New Zealand and American COI clades in parsimony analyses is six steps (0.95% of the 626 nt fragment). Although the resulting age estimate of 0.625 myr for the *O. equestris*/*O. aupouria* disjunction is undoubtedly crude, it is over two orders of magnitude less than the vicariant separation of New Zealand from Gondwanaland (Weissel & Hayes, 1977).

The *Ostreola equestris*/*O. aupouria* geographic disjunction is but one of three such cases involving tip taxa in the brooding oyster 16S gene tree (Fig. 3); the other two involve *Ostrea edulis*/*O. angasi* and *Crypytostrea permollis*/*Ostrea puelchana* and are discussed in Jozefowicz & Ó Foighil (1998). Although anthropogenic transoceanic oyster introductions have occurred on numerous occasions (Dinamani, 1971; Edwards, 1976; Buroker et al., 1979; Chew, 1990; Carlton & Mann, 1996; Boudry et al., 1998; Ó Foighil et al., 1998), we can, with some confidence, rule out such historic transfers among the New Zealand/American study populations (Fig. 4). This conclusion is based on their lack of shared COI haplotypes and on their reciprocal monophyly (Fig. 5), a phylogenetic relationship that is characteristic of populations that have not experienced evolutionary recent gene flow (Avise, 2000). It is possible, however, that such an event may have occurred involving yet-to-be-sampled, genetically differentiated portions of either species' ranges – according to Harry (1985), *O. equestris* occurs from North Carolina to Argentina.

Genetic Structuring of Gulf/Atlantic *Ostreola equestris* and *Crassostrea virginica*

Genetic characterization of near-shore marine taxa found on either flank of the Floridian peninsula have revealed cryptic phylogenetic disjunctions among diverse Gulf-Atlantic Carolinian faunal elements (Saunders et al., 1986; Bert, 1986; Avise et al., 1987; Bert & Harrison, 1988; Dillon & Manzi, 1989; Brown & Wolfingbarger, 1989; Cunningham et al., 1991; Sarver et al., 1992; Cunningham & Collins, 1994; Felder & Staton, 1994; Bert & Arnold, 1995; Duggins et al., 1995; Ó Foighil et al., 1996; Schizas et al., 1999; Avise, 2000; Collin, 2001, 2002), with by far the most intensively studied exemplar being the American oyster *Crassostrea virginica* (Reeb & Avise, 1990; Karl & Avise, 1992; McDonald et al., 1996; Hare & Avise, 1996, 1998; Hare et al., 1996). *Ostreola equestris* occurs in micro-sympatry with *C. virginica* throughout regional estuaries, although prior research has shown that *O. equestris* tends to be abundant only at high salinity portions of estuaries (Hoese, 1960). Surprisingly, our *O. equestris* mt COI data (Fig. 5, Table 2) show that this oyster species differs from *C. virginica*, and from a large fraction of the regional marine biota, in lacking a Gulf/Atlantic mt genetic disjunction. Absence of genetic structuring among Gulf and Atlantic populations is not unique to *O. equestris* (Gold & Richardson, 1998; Avise, 2000); however, our results indicate that these two co-occurring oyster species have experienced significantly different regional histories.

Another discrepancy among the two oyster mt datasets concerns the relative topological definition of Gulf and Atlantic populations. Beckenbach (1994) performed a cladistic analysis of Reeb & Avise's (1990) extensive (N = 232) *C. virginica* mt RFLP dataset and found that both Gulf and Atlantic populations were dominated by one or two common haplotypes. These occupied central positions in their respective clades and were separated by single steps from a large number of terminally positioned rare haplotypes. Our Gulf (Cedar Key) and Florida Keys samples of *Ostreola equestris* showed (either separately or jointly) essentially a similar topology; however, the Atlantic (Skidaway River) sample did not (Fig. 5). In the absence of significant homoplasy, the relative lengths of individual branches within a molecular phylogenetic tree topology are rough proxies for evolutionary time. In this context, it is interesting to note the markedly longer collective branch lengths interconnecting *Ostreola equestris* Atlantic haplotypes relative to the truncated area of the COI topology occupied by Gulf and Florida Keys haplotypes (Fig. 5). This topological distinction is consistent with an older evolutionary history for this species in the Atlantic section of its present-day regional range. The compact star-like haplotypic topology produced by Gulf (Cedar Key) and Florida Keys COI genotypes (Fig. 5) is characteristic of a population founded more recently by one ancestral type, presumably represented by the numerically predominant, topologically central, well-connected (Castelloe & Templeton, 1994) haplotype AFG1, found in all three study populations. Such a topology is also indicative of populations that have experienced a phase of rapid demographic growth, a process associated with lowered stochastic elimination of novel/rare lineages (Avise et al., 1984; Slatkin & Hudson, 1991; Moritz, 1996).

Our mt COI data for the three study populations of *Ostreola equestris* paint a regional history that differs in important respects from that of *Crassostrea virginica* and also from a large fraction of the local marine biota. The dominant regional theme is the presence of a Gulf-Atlantic phylogeographic break characterized by considerable geographic concordance in genetic structuring across diverse faunistic elements (Avise, 2000). This implies a coherent spatial patterning of vicariance and secondary contact events. In contrast, *O. equestris* shows no evidence of a vicariant imprint and our results imply that its present day Gulf/Atlantic distribution has been achieved by range extension from source Atlantic populations followed by a demographic growth pulse in the new Florida Keys/Gulf of Mexico habitats.

Shell Phenotype Variation in *Ostreola equestris*

Though forearmed with an awareness of the fabled xenomorphism of oysters, we were surprised at the extent to which *O. equestris*, the most commonly encountered ostreid in the Florida Keys, exhibited a multitude of shell phenotypes – a repertoire far from exhausted by our limited presentation in Figure 6. This facility is also a characteristic of *Ostrea aupouria*, its New Zealand sister taxon (Dinamani & Beu, 1981). Although genetic characterization is a reliable method for distinguishing co-occurring oyster species with overlapping shell morphs, the presence of a distinct anal appendage in *O. equestris* (Harry, 1985; but not all are digitiform) and in *O. aupouria* (Dinamani & Beu, 1981) is also particularily useful in this regard. It is unclear to what degree the phenotypic variation we observed in *O. equestris* reflects populational allelic diversity and/or local micro-environmental parameters, or what contribution this plasticity makes to the local ecological success of this small species – the numerically dominant Florida Keys oyster.

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